Notes and Comments

Competition and Dispersal in *Pseudomonas aeruginosa*

Tiffany B. Taylor* **and Angus Buckling**

Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

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abstract: Dispersal plays a crucial role in a range of evolutionary and ecological processes; hence there is strong motivation to understand its evolution. One key prediction is that the relative benefits of dispersal should be greater when dispersing away from close relatives, because in this case dispersal has the additional benefit of alleviating competition with individuals who share the same dispersal alleles. We tested this prediction for the first time using experimental populations of the opportunistic pathogen *Pseudomonas aeruginosa*. We measured the fitness of isogenic genotypes that differed only in their dispersal behaviors in both clonal and mixed populations. Consistent with theory, the benefit of dispersal was much higher in clonal populations, and this benefit decreased with increasing growth rate costs associated with dispersal.

Keywords: kin selection, experimental evolution, bacteria, virulence, type IV pili.

Introduction

Dispersal plays a crucial role in a range of evolutionary and ecological processes; hence there is strong motivation to understand its evolution (Gadgil 1971; Dieckmann et al. 1999; Clobert et al. 2001, 2004; Bullock et al. 2002). Theoretical work suggests that dispersal is likely to be favored by selection when the environment varies in time (Van Valen 1971; McPeek and Holt 1992) and if it reduces inbreeding depression (Bengtsson 1978) and kin competition (Hamilton and May 1977). Selection for dispersal can in turn be opposed if it is associated with any costs, such as increased mortality or reduced reproduction (Rousset and Gandon 2002). Here we carry out an experimental study using bacteria to simultaneously test for the first time the qualitative predictions that competition with individuals who share the same dispersal alleles (Hamilton and May 1977; Comins et al. 1980; Taylor and Frank 1996; Gandon and Michalakis 1999) should increase the benefit of dispersal, whereas decreased mean growth rates away from the home patch should decrease the benefit.

Evidence for the importance of kin competition in determining dispersal patterns has been found in both experimental and field studies and in a wide range of taxa, including mammals (Bollinger et al. 1993), reptiles (Cote et al. 2007), insects (Kasuya 2000), and birds (Strickland 1991). We explicitly investigated the importance of kin competition and dispersal costs on the evolution of dispersal in the opportunistic pathogen *Pseudomonas aeruginosa*. This bacterium possesses a range of motility mechanisms, presumably to cope with the wide range of environments in which it inhabits. *Pseudomonas aeruginosa* expresses two surface organelles that aid motility: a single polar flagellum and retractable polar type IV pili. In addition, it is also able to secrete a lipid-based biosurfactant called rhamnolipid (Mattick 2002). This enables the bacterium to twitch (using type IV pili as grappling hooks [Henrichsen 1972]), swim (using flagella [Bai et al. 2007]), or swarm (comparable to gliding, using rhamnolipids coupled with flagella [Köhler et al. 2000; Caiazza et al. 2005]), depending on the environment. We used isogenic mutants of *P. aeruginosa* strain PAO1, which showed high (disperser) or low (nondisperser) swimming dispersal. These phenotypes were achieved by using transposon mutants defective in type IV pili function. The dispersers were unable to express type IV pili (*pilA* mutant), and the nondispersers constitutively expressed them (*pilU* mutant). Within a semisolid substrate, these pili cause drag and reduced the efficiency of flagella-mediated swimming motility, resulting in a smaller colonization area.

We manipulated the degree of kin competition by inoculating strains as either clonal or mixed populations into homesites, with the former being the high kin competition treatment. Note that nonkin differ from each other only with respect to their dispersal strategy, as is assumed in theoretical studies (Hamilton and May 1977; Gandon and Michalakis 1999). The relative fitness of dispersers versus nondispersers was determined by calculating the ratio of the total number of each cell type in both the homesite and the surrounding area after replication and dispersal.

* Corresponding author; e-mail: tiffany.taylor@zoo.ox.ac.uk.

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This measure of fitness for the clonal and mixed populations therefore indicates how dispersal strategies would likely evolve in metapopulations under conditions of high and low relatedness, respectively. These simple competition assays were carried out with variable costs of dispersal; the latter was manipulated by reducing nutrient availability away from the home patch.

Experimental Procedures

Strain Details and Growth Conditions

Two transposon mutants defective in type IV pili and generated from a wild-type strain of *Pseudomonas aeruginosa* (PAO1) were used: a *pilU* (nondisperser) mutant, which is able to express but unable to retract pili (hyperpiliated), and a *pilA* (disperser) mutant, which is absent of pili. Cultures for plate colonization were grown overnight in a homogeneous (shaken) environment (0.9 *g*) at 37°C in 30-mL universal vials containing 6 mL fresh medium (King's B: 20 g/L protease peptone, 10 g/L glycerol, 1.5 g/L potassium phosphate, and 1.5 g/L magnesium sulphate heptahydrate).

Treatment Conditions

Overnight cultures (shaken at 0.9 *g* at 37°C) were vortexed thoroughly, and 10^7 cells of dispersers, nondispersers, or a 1 : 1 mixture of the two were pipetted into 25 mL semisolid King's B medium agar (0.6% w/v) plates (which were briefly dried in a flow hood for 20 min) just below the surface line so the point of colonization could be located. Plates were left in a humid incubator for 42 h at 37°C. Within the mixed treatment, each genotype was at half the cell density compared with clonal treatments. In order to understand the effects of density on dispersal, a range of dilutions (1 in 2, 1 in 10, 1 in 10^3, and 1 in 10^6) were also used to inoculate soft agar plates using the same method described above (distance traveled from inoculation site was determined as the radius calculated from total colony area). Finally, to ensure that any growth inhibition of the disperser within mixed treatments was only due to the effects of competition, a soft agar plate inoculated with a 1 : 1 mixed treatment was left for 138 h at 37°C. Control conditions where dispersal behavior was not under selection were created by growing bacteria at 37°C in 6 mL liquid KB shaken at 0.9 *g* as well as in 0.6% w/v KB agar, where the bacteria were distributed evenly throughout the plate. Six replicates of disperser, nondisperser, and mixed treatments were set up in three experimental blocks.

Data Collection

The area covered by the bacteria was calculated (using Analyzing Digital Images software; http://mvh.sr.unh.edu/software/software.htm), and nine samples were taken from each plate to calculate cell densities. These samples were taken using a 1-mL pipette (Finnpipette) at 5-mm intervals from the point of colonization along the radius line of the colony. Samples were placed in an eppendorf containing 1 mL M9 solution (1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). The pipette tip was washed thoroughly in the solution, and the eppendorf was subsequently vortexed. Bacteria were then plated onto KB agar at an appropriate dilution. Plates were incubated overnight and the number of colony-forming units counted. Morphological differences between disperser and nondisperser colony-forming units were sufficient to allow visual differentiation between strains (with the former being noticeably larger and having less defined colony edges). At the very edge of the nondisperser colonies, the morphology of the colony-forming units increased in size, although they could still be readily distinguished from the dispersers. Total cell numbers on the plate were estimated by scaling up the colony-forming unit counts from the area of the pipette tip. The relative fitness of dispersers versus nondispersers was determined by calculating the ratio of the total number of each cell type in both the homesite and in the surrounding agar after 48 h replication and dispersal. For the clonal populations, this ratio was calculated between pairs of plates (from the same experimental block), whereas for the mixed population, the ratio was calculated within each plate.

Imposing a Cost to Dispersal

Twenty-five milliliters of semisolid King's B medium agar (0.6% w/v) was allowed to dry for approximately 10 min, after which 0%, 25%, 50%, 75%, or 100% of the agar was cut away (leaving a high-nutrient zone of 1.0-cm diameter around the inoculation site to ensure only those dispersing were incurring a cost) and replaced with 0.6% w/v agar that did not contain any nutrients (6 g/L agar and water). Plates were dried for an additional 10 min and inoculated with 10^7 of clonal or mixed cells. Plates were left in a humid incubator for 42 h at 37°C. Sampling techniques were as described above, except 17 samples were taken from each plate (one from the central colonization point and eight more at regular 5-mm intervals from the area of high-nutrient and no nutrient agar). One replicate of disperser, nondisperser, and the mixed treatments were set up in each of the five cost groups.

Results and Discussion

We measured the relative fitness of isogenic dispersing and nondispersing genotypes of *Pseudomonas aeruginosa* under conditions that allowed swimming dispersal in both clonal and mixed populations. Dispersing genotypes grew in the area in which they were initially inoculated and also dispersed away and replicated outside this area. By contrast, nondispersing mutants were largely limited to growth within the initial inoculation area (fig. 1*A*; GLM: the effect of genotype on dispersal area, $F_{1,6} = 128.48$, $P < .0001$). Increased dispersal had a clear fitness advantage in clonal populations, with cell density counts of the dispersing genotype after 42 h growth and dispersal approximately 16-fold higher than that of the nondispersing genotype (fig. 1*A*, 1*C*; Wilcoxon test, $P = .036$). The advantage of dispersal was presumably that the initial site of inoculation deteriorated in quality through time as a result of resource depletion and the buildup of toxins. This is consistent with the general prediction that temporal variation (in this case, local environmental deterioration) favors dispersal (Van Valen 1971; McPeek and Holt 1992; Friedenberg 2003). By contrast, when both dispersers and nondispersers were coinoculated into the center of the agar plate, the dispersers achieved only a twofold higher final density relative to the nondispersers (fig. 1*B*, 1*C*; Wilcoxon, $P = .059$). The greater advantage to the dispersing genotype in clonal versus mixed populations (Mann-Whitney, $P = .0051$) suggests that the advantage of dispersal will be greatest under conditions of high within-patch relatedness in metapopulations, as predicted by theory (Hamilton and May 1977; Taylor and Frank 1996; Gandon and Michalakis 1999; Rousset and Gandon 2002).

The fitness of dispersing genotypes is predicted to increase with increasing kin competition because dispersal reduces competition within the home patch (Hamilton and May 1977; Gandon and Michalakis 1999; Rousset and

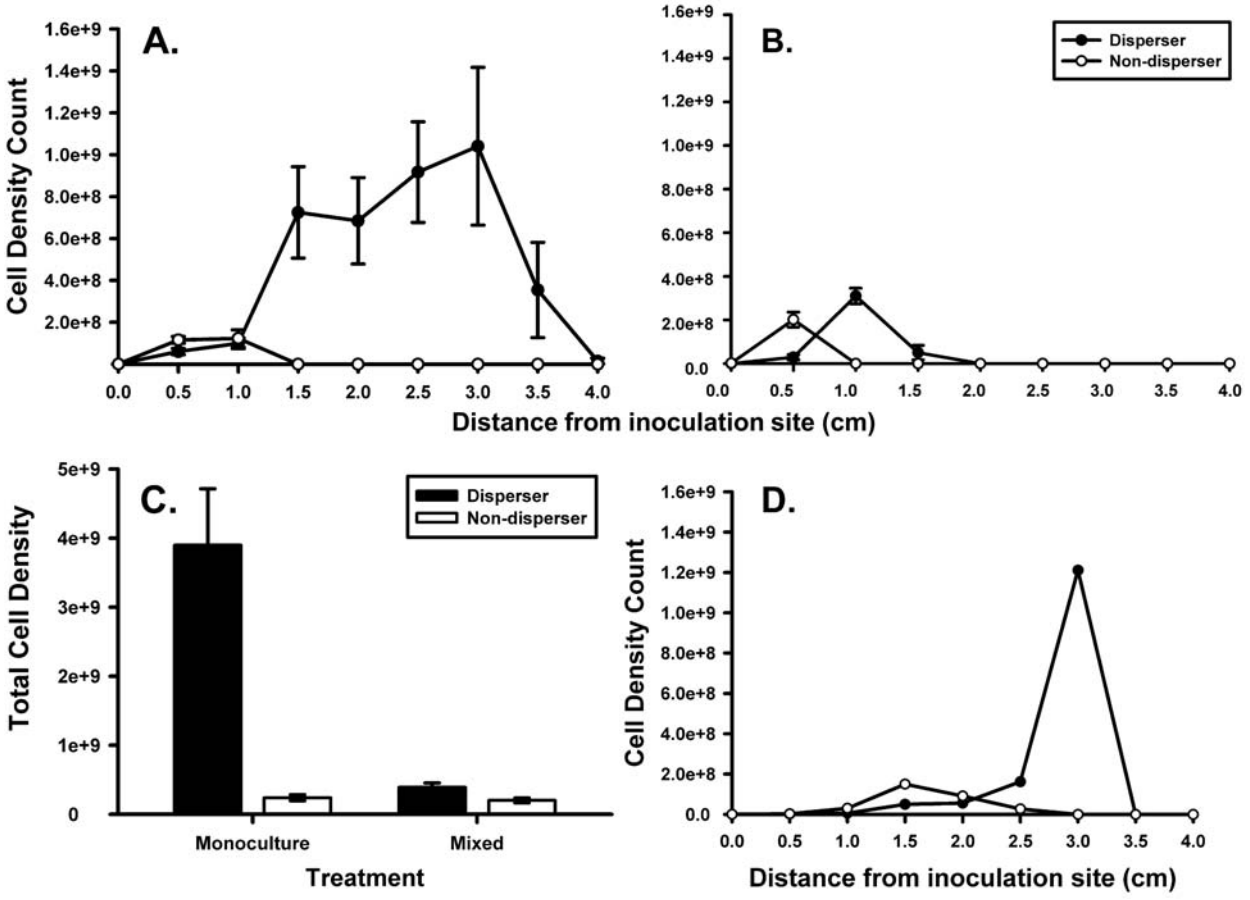

Figure 1: Average cell density of dispersers and nondispersers between treatments (±SEM). Monocultures of dispersers and nondispersers (*A*) and a 1 : 1 mixture (*B*) were grown in KB agar (0.6% w/v) for 42 h. Nine samples were taken along the radius of the colony and the total cell density over the plate estimated (*C*; presented on a log scale). Mixtures were also left for 138 h (*D*).

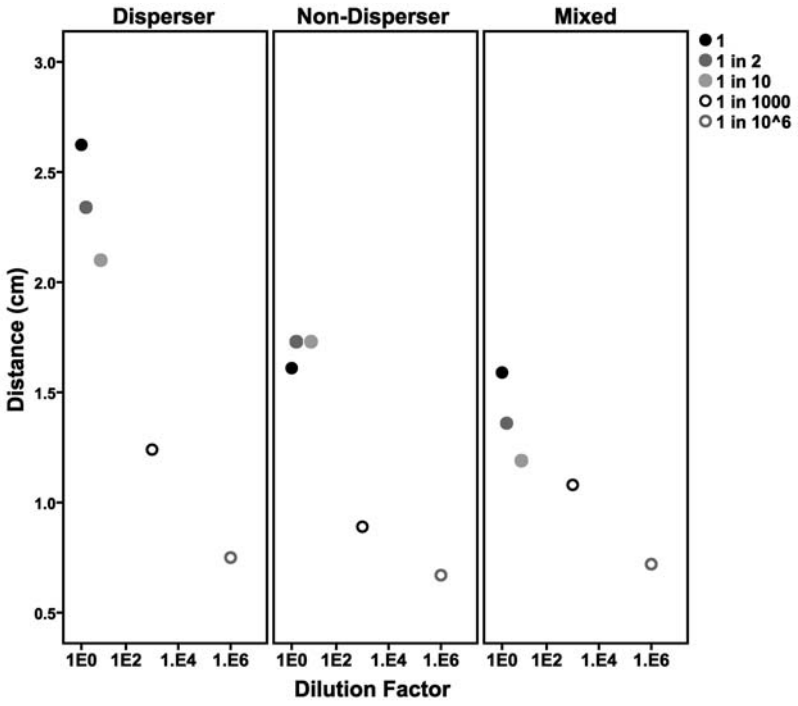

Figure 2: Dispersal distance depends on cell number. A number of dilutions (1 in 10^0, 1 in 2, 1 in 10^1, 1 in 10^3, and 1 in 10^6) of each treatment group (monocultures of dispersers, nondispersers, and a 1 : 1 mixture of the two) were used to inoculate KB agar plates (0.6% w/v). After 42 h, the distance traveled from the inoculation site was measured. The results clearly show that fewer inoculating cells reduce subsequent dispersal distance.

Gandon 2002). Consistent with this prediction, we found that the nondisperser had a much stronger competitive effect than the disperser within the home patch: the density of the dispersing genotype was reduced by more than threefold within the inoculation site by the presence of the nondispersing genotype ($t = 2.38$, $P = .038$), whereas the density of the nondispersing genotype was not altered by the presence of the dispersing genotype ($t = 1.08$, $P = .3$; *t*-test comparing relative density reduction caused by the competing genotype of dispersing vs. nondispersing genotype: $t = 2.86$, $P = .017$). It was necessary to address the possibility that reduced competitiveness of the disperser in the inoculation site was not in fact the result of dispersal but instead was because the disperser was an intrinsically worse competitor than the nondisperser. To resolve this, we measured relative growth rates of the genotypes in environments where dispersal ability was expected to have little or no impact on fitness. First, we competed the disperser and nondisperser in shaken tubes, where swimming dispersal would be irrelevant compared with mechanical dispersal. Second, we competed the genotypes where bacteria were evenly inoculated throughout soft agar; hence moving from one colonized "patch" would simply result in entering another. We found no difference in relative growth rate between the disperser and the nondisperser under these conditions (in shaken liquid: Wilcoxon, $P = .933$; evenly distributed throughout soft agar: Wilcoxon, $P = .14$).

A potentially confounding feature of our experimental design is that the disperser colonized a significantly smaller distance in the presence versus in the absence of the nondisperser (fig. 1*B*; $t = 5.52$, $P = .003$). Despite this, the dispersers always dispersed beyond the range of the nondispersers in mixed populations ($F_{1,10} = 121.0$, $P < .0005$) and showed a recovery of cell number beyond the range of the nondispersers when left for 138 h (rather than 42 h; fig. 1*D*). Reductions in dispersal distance in the mixed populations might have arisen if nondispersers physically prevented dispersal of the dispersers, in which case the mixed treatment might have altered not only the strength of kin competition but also dispersal rates of individual bacteria. However, colonization distance of bacteria is determined by both growth and dispersal (Henrichsen 1972); hence these data are also consistent with a reduction in density of the disperser. To distinguish between these possibilities (physical prevention of dispersal and reduced density), we determined how colonization distance changed as a function of inoculation density. Consistent

with the latter hypothesis, colonization distance decreased with decreasing density in both clonal and mixed populations (fig. 2; main effect of density: $F_{1,13} = 8.65$, $P = .011$). It is of particular significance that colonization distance decreased with density in mixed populations: if reductions in dispersal resulted from physical interference of the disperser by the nondisperser, colonization distance should have increased with lower inoculation densities.

Within natural populations, there are likely to be costs associated with dispersal (Ronce 2007) via direct resource consumption (e.g., in the form of energy expenditure during travel [Weber and Stilianakis 2007] or in the manufacture of necessary motility structures [Zera and Denno 1997]), increased risk of mortality (Hanski et al. 2000), or reduced fitness due to an unsuitable destination habitat (McPeek and Holt 1992). We wanted to introduce costs of dispersal into our experimental design and observe how it affected the fitness of the dispersal strategies. This was achieved by introducing low-nutrient areas into the environment, on which bacterial growth was severely limited. This area encompassed 0%, 25%, 50%, 75%, or 100% of the total area available for colonization beyond 5 mm from the point of inoculation. The results are shown in figure 3. There was an overall decline in fitness of the disperser with increasing costs in both clonal (Spearman rank correlation: $r = 1$, $P < .001$) and mixed ($r = 0.9$, $P = .037$) populations. Moreover, the costs of dispersal that resulted in nondispersers having a selective advantage are greater in clonal compared with mixed populations (treatment × cost interaction: $F_{1,6} = 103.75$, $P < .0005$), as predicted by theoretical work (Gandon and Michalakis 1999; Rousset and Gandon 2002). Specifically, the relative fitness of the disperser dropped below that of the nondisperser when greater than 93% and 71% of the environment was unable to support growth in clonal and mixed populations, respectively.

Our experiments inevitably do not capture many of the details developed in theoretical studies, and this may limit the interpretation of our study. First, we infer evolution only within a metapopulation from our single-patch competition experiments; hence we have been unable to investigate feedbacks between dispersal, relatedness, and variation in colonization dynamics (Comins et al. 1980; Gandon and Michalakis 1999; Rousset and Gandon 2002). However, we argue that the fitness of different dispersal strategies within clonal and mixed populations is a good proxy of how dispersal strategies would likely evolve in metapopulations under conditions of high and low relatedness, respectively. Second, dispersal in this experimental context is also dependent on growth. As a result, we cannot unequivocally rule out the possibility that dispersal is also limited by physical inhibition between genotypes, although this seems unlikely given that reducing density (and hence opportunities for physical contact) decreased, rather than

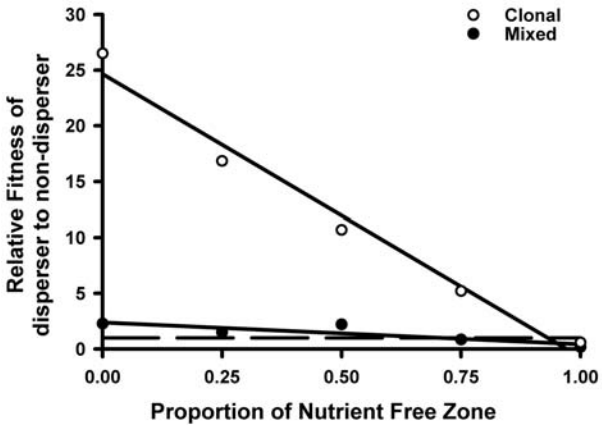

Figure 3: Imposing a cost to dispersal. A cost to dispersal was imposed by introducing nutrient-free areas over 0%, 25%, 50%, 75%, or 100% of the agar plates. The results showed the relative fitness of the disperser to the nondisperser in a clonal population (*open circles*) to be consistently greater (except when 100% is a nutrient-free area); however, in a mixed population (*solid circles*), the relative fitness of the disperser falls below that of the nondisperser when 71% of the environment was devoid of nutrients and unable to support bacterial growth. The dashed line indicates a relative fitness of 1, that is, when the fitness of the disperser and the nondisperser are equivalent.

increased, colonization distance in mixed populations. Third, we did not allow the dispersal phenotypes to evolve as a result of mutations that are generated during the experiment but instead rely on simple competition experiments between two defined and widely contrasting strategies. This inevitably results in strong selection operating between genotypes in our experimental setup, whereas most kin selection models of the evolution of dispersal assume, for analytical ease, very weak selection (but see Gardner et al. 2007). Finally, as shown in figure 1, the results are likely to quantitatively vary if densities are measured after different time points: allowing a longer time to disperse increases the relative fitness of the dispersing genotype in the mixture. However, there is no reason why this should qualitatively affect the results, since longer dispersal time also benefited the dispersing genotype in clonal populations, to the extent that it completely colonized the whole agar plate.

These results may have implications for understanding the evolution of virulence (the amount of host damage caused by the parasite) of *P. aeruginosa* and similar opportunistic pathogens. Dispersal has been linked to virulence both directly (via the colonization of host tissues [Drake and Montie 1988; Feldman et al. 1998]) and indirectly (via the production of biofilms [Jenkins et al. 2005; Josenhans and Suerbaum 2002]). Clinical research has identified natural pili variants in the airways of cystic fi-

brosis sufferers infected with *P. aeruginosa* (Head and Yu 2004; Smith et al. 2006), with the general trend that motility function tends to be lost over time (Mahenthiralingam et al. 1994). This loss could be due to a survival advantage, such as a greater resistance to phagocytosis (Mahenthiralingam et al. 1994) and bacteriophages (Bradley 1974; Brockhurst et al. 2005), but the social environment may also impose selection for changes in motility.

Acknowledgments

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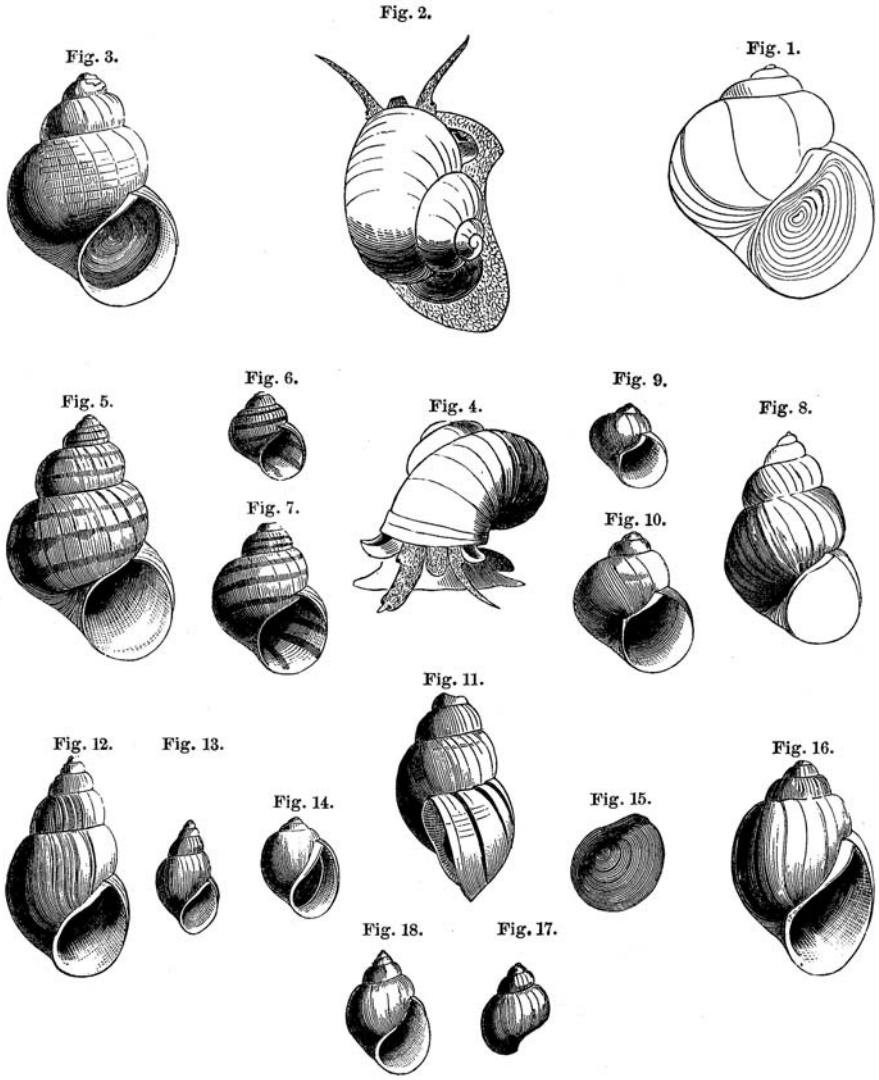

1, Vivipara georgiana; 2, Vivipara intertexta Say, crawling; *3, V. intertexta; 4, V. intertexta; 5, Vivipara contectoides; 6, V. contectoides; 7, V. contectoides; 8, Vivipara subpurpura; 9, V. subpurpura; 10, V. subpurpura; 11, Melantho decisa,* the only species found in New England; *12, Melantho coarctata; 13, M. coarctata; 14, Melantho ponderosa,* young; *15, Vivipara georgiana; 16, M. ponderosa,* young; *17, Melantho integra; 18, M. integra.* From "Our Common Fresh-Water Shells" by E. S. Morse (*American Naturalist,* 1869, 3:530-535).